λ_{Em} = 517 nM) and varied bleomycin. Bleomycin concentrations: 200 nM (o), 100 nM (❑), 50 nM (◇), 25 nM (Δ), 12.5 nM (·), 5 nM (■) and 2.5 nM (▲). **(b)** The observed DNA cleavage over time of an assay containing a constant 3.2 nM break light A at 37°C (40 mM Tris HCl, 2.5 mM ascorbate, pH 7.5; λ_{Ex} = 485 nm, λ_{Em} = 517 nM) and varied MPE. Fe(II) concentrations: 8 μM (o), 4 μM (❑), 2 μM (◇), 1 μM (Δ), 500 nM (·), 250 nM (■) and 125 nM (▲). **(c)** The observed DNA cleavage over time of an assay containing a constant 32 nM break light A at 37°C (40 mM Tris HCl, 2.5 mM ascorbate, pH 7.5; λ_{Ex} = 485 nm, λ_{Em} = 517 nM) and varied MPE. Fe(II) concentrations: 50 nM (o), 125 nM (❑), 250 nM (◇), 500 nM (Δ), 1 μM (·) and 2 μM (■). **(d)** The observed DNA cleavage over time of an assay containing a constant 32 nM break light A at 37°C (40 mM TrisHCl, 2.5 mM ascorbate, pH 7.5; λ_{Ex} = 485 nm, λ_{Em} = 517 nM) and varied Fe^{+2}-EDT A. Fe(II) concentrations: 12.5 μM (o), 6.3 μM (❑), 3.1 μM (◇), and 1.3 μM (Δ).

At page 12, the paragraph beginning on line 1:

Figure 6A is a graph of the UV-visible absorption spectra of purified mbp-CalC. The purified mpb-CalC was analyzed in the following solution: 52 μM mpb-CalC; 10 mM Tris-HCl, pH 7.5). The inset shows the results of low temperature (4.3 K) the X-band EPR analysis of CalC. 250 μM mpb-CalC containing 0.5 mol Fe per mol CalC was analyzed in 10 mM Tris-HCl, pH 7.5. The spectrometer settings were as follows: field set = 2050 G; scan range = 4,000G; time constant = 82 s; modulation amplitude =16 G; microwave power = 31 μW; frequency = 9.71 Ghz; gain = 1000; determined spin quantitation = 90 ±10 μM Fe.

At page 12, the paragraph beginning on line 9:

Figure 6B is a photograph of an ethidium bromide stained agarose gel. Lane A: calicheamicin, no DTT; lane B: DTT, no calicheamicin; lane C: DTT and calicheamicin; lane D: DTT, calicheamicin, and mbp; lane E: calicheamicin, DTT, and *apo*-mbp-CalC (which lacks the Fe cofactor); lane F: DTT, calicheamicin, and mbp-CalC; and lane G: calicheamicin, DTT, and *apo*-mbp-CalC, preincubation with 1 mM $FeSO_4$ (Fe^{+2}) or $FeCl_3$ (Fe^{+3}) prior to the activity assay.

At page 12, the paragraph beginning on line 15:

Figure 7 is a schematic diagram of the first continuous assay for enediyne-induced DNA cleavage, the Molecular Break Lights. The solid lines represent covalent bonds, dashed lines represent hydrogen bonding, letters represent arbitrary bases, the gray shaded ball represents the fluorophore (FAM: fluorescein), the black ball represents the corresponding quencher (DABCYL:4-(4-'demethylaminophenylazo)-benzoic acid) and the dashed wedges represent fluorescence.

At page 12, the paragraph beginning on line 21:

Figure 8 shows the direct in vitro inhibition of calicheamicin-mediated DNA cleavage using the break light assay. 3.6pM break light A is coincubated with 3.5nM calicheamicin with increasing amounts of CalC. Complete inhibition of calicheamicin is achieved with roughly 2-fold excess of CalC. CalC has no effect on esperamicin-induced cleavage of DNA.

A1 Cont'd

At page 13, the paragraph beginning on line 30:

A2

Generally, molecular beacons operate by a separation of the fluorophore-quencher pair resulting in a corresponding fluorescent signal. Molecular break lights, as illustrated in the **FIG. 7**, operate through cleavage of the stem by an enzymatic or non-enzymatic nuclease activity resulting in the separation of the fluorophore-quencher pair and corresponding fluorescent signal. In **FIG. 7**, the molecular break lights contain either a preferred calicheamicin recognition site (bold-faced, TCCT) or the BamHI recognition site (bold-faced, GGATCC). The predicted cleavage sites are illustrated by arrows.

At page 22, the paragraph beginning on line 19:

A3

Enediyne-catalyzed cleavage was also assessed. Previous assays for enediyne cleavage of DNA relied upon discontinuous assays using radioactive DNA probes, electrophoresis and subsequent phosphoimager analysis. In contrast, by using the molecular break lights of the present invention, one can directly follow the extent of DNA cleavage by a specific enediyne in real time with high sensitivity. Under the

conditions described, this assay allows the detection of calicheamicin in the pM range. This sensitivity compares to that of the biochemical induction assay (BIA), the method of choice in detecting DNA-damaging agents. *See, e.g.*, Roy, K.B., *et al. Anal. Biochem.* 220: 160-164 (1994). Furthermore, the sensitivity can be significantly enhanced by simply increasing the concentration of the molecular break light in the assay as demonstrated with the iron-dependent agents. The observed maximum fluorescence obtained upon cleavage of 3.2 nM break light A with either calicheamicin or esperamicin was identical to that observed with DNaseI, consistent with complete degradation of the oligonucleotide. As controls, incubation of molecular break light A with either DTT or enediyne alone revealed no change in fluorescence. Furthermore, although there is some debate regarding the "specificity" of calicheamicin, break light B was cleaved by calicheamicin at an identical rate. This supports the view that the specificity of calicheamicin is more dependent upon context and perhaps less so on DNA sequence. It should also be noted that calicheamicin leads to predominately double-stranded cleavage while esperamicin provides single-stranded nicks and the current molecular break light assay can not distinguish these two phenomena.

A3 cont'd

At page 23, the paragraph beginning on line 23:

A4

Cleavage catalyzed by Fe^{+2}-dependent agents was assessed. To further demonstrate the utility of molecular break lights, the ability to assess DNA cleavage catalyzed by Fe^{+2}-dependent agents was investigated. The agents selected include the natural metabolite from *Streptomyces verticillus*, bleomycin, **FIG 1c**, and two DNA-footprinting reagents, methidiumpropyl-Fe- EDTA (MPE), **FIG. 1d**, and Fe-EDTA, **FIG. 1e**. While the precise mechanism of DNA cleavage by bleomycin is still controversial, MPE and Fe^{+2}-EDTA cleave DNA via the generation of diffusable hydroxy radicals which ultimately contribute to oxidative DNA cleavage. Of these three, bleomycin also contains a strong minor groove binding constituent while MPE carries a DNA intercalator. As with the previous enediyne assays, reported assays for cleavage by these agents have all relied upon discontinuous systems and thus, molecular break lights should present an obvious advantage. **FIG. 5** illustrates agent concentration dependent cleavage of break light A. Under the conditions described, this assay allows the detection of bleomycin in the nM range which represents a slight

increase in sensitivity over the biochemical induction assay (BIA) and reiterates the power of this assay to detect the production of naturally-produced DNA-damaging agents.

At page 24, the paragraph beginning on line 8:

To increase the sensitivity for the less efficient reagent Fe^{+2}-EDTA, oligo concentration was increased 10-fold (32 nM), **FIG. 5d**. As a comparison, MPE was also examined at this higher molecular break light concentration, **FIG. 5c**. Finally, while ascorbate is critical for efficient DNA-cleavage by MPE and by Fe^{+2}-EDTA, the addition of ascorbate did not affect DNA-cleavage by bleomycin.

A4 Cont

At page 24, the paragraph beginning on line 29:

As indicated in **FIG. 6**, no DNA cleavage was observed in the absence of DTT or calicheamicin (lanes a and b), while efficient cleavage was demonstrated in the presence of DTT and calicheamicin (lane c). As expected, the addition of mbp-CalC completely inhibited calicheamicin-induced DNA cleavage (lane f) while the addition of mbp alone (lane d) as a control, failed to inhibit calicheamicin-induced DNA cleavage. Furthermore, preincubation of mbp-CalC with DTT (not shown), or *apo*-mbp-CalC (lacking the Fe cofactor)(lane e), also failed to inhibit calicheamicin-induced DNA cleavage. However, the addition of Fe^{+2} or Fe^{+3} to the *apo*-mbp-CalC assay could reconstitute CalC activity (lane g). Reconstitution of *apo*-mbp-CalC was accomplished by preincubation with 1 mM $FeSO_4$ (Fe^{+2}) or $FeCl_3$ (Fe^{+3}) prior to the activity assay as previously described.

A5

At page 25, the paragraph beginning on line 8:

CalC inhibition of calicheamicin mediated DNA cleavage was examined. Two molecular break lights for the experiments are shown in **FIG. 2**. Break light A was comprised of a 10-base pair stem which contained the known calicheamicin recognition sequence 5'-TCCT-3', while break light B carried the *BamHI* endonuclease recognition sequence 5'-GGATCC-3'. The length of break light B also considered the requirement of a 3 base pair overhang required for *BamHI* recognition and the stem of break light A was adjusted to a comparable length and melting temperature. The loop of both probes consisted of a T_4 loop to ensure non-

hybridizing interactions. The 5'-fluorophore of both probes was fluorescein (FAM, absorbance$_{max}$ = 485 nm, emission$_{max}$ = 517 nm) while the corresponding 3'-quencher was 4-(4'-dimethylaminophenylazo)benzoic acid (DABCYL). **FIG. 7** is a representation of the cleavage of break light A by calicheamicin and of break light B by *BamH*I.

At page 25, the paragraph beginning on line 21:

As illustrated in **FIG. 8**, CalC directly inhibits of calicheamicin-mediated DNA cleavage in the break light assay. 3.6pM break light A is coincubated with 3.5nM calicheamicin with increasing amounts of CalC. Complete inhibition of calicheamicin is achieved with roughly 2-fold excess of CalC. CalC has no effect on esperamicin-induced cleavage of DNA (data not shown).

At page 31, the paragraph beginning on line 12:

Under the conditions described, this assay allows the detection of calicheamicin in the pM range. No change in fluorescence was observed in the controls, incubation of molecular break light A with either DTT or enediyne alone. Furthermore, break light B was cleaved by calicheamicin at a rate identical to that of break light A.

At page 32, the paragraph beginning on line 11:

FIG. 5A illustrates agent concentration dependent cleavage of break light A by Blenoxane. Under the conditions described, this assay allows the detection of bleomycin in the nM range. Although ascorbate is critical for efficient DNA-cleavage by MPE and by Fe^{+2}-EDTA, the addition of ascorbate did not affect DNA-cleavage by bleomycin.

At page 32, the paragraph beginning on line 25:

In a first assay, 3.2 nM break light A was incubated in 40 mM Tris HCl and 2.5 mM ascorbate at 37°C and pH 7.5. Cleavage was initiated by addition of MPE/Fe(II) in a 1.2:1 molar ratio to various concentrations. Final Fe(II)

concentrations were 8 μM, 4 μM, 2 μM, 1 μM, 500 nM, 250 nM, and 125 nM. Results are shown in **FIG. 5B**.

At page 32, the paragraph beginning on line 30:

In a second assay, 32 nM molecular break light A was incubated in 40 mM Tris and 2.5 mM sodium ascorbate at pH 7.5 and 37°C. Cleavage was initiated by addition of MPE/Fe(II) in a 1.2:1 molar ratio to various concentrations. Final Fe(II) concentrations were 50 nM, 125 nM, 250 nM, 500 nM, 1 μM, and 2 μM. Results are shown in **FIG. 5C**.

At page 33, the paragraph beginning on line 5:

In a third assay, 32 nM break light A (calicheamicin-specific molecular break light oligonucleotide) was incubated in 40 mM Tris and 2.5 mM sodium ascorbate at pH 7.5 and 37°C. Cleavage was initiated by addition of EDTA/Fe(II) in a 2:1 molar ratio to various concentrations. Final Fe(II) concentrations were 12.5 μM, 6.3 μM, 3.1 μM, and 1.3 μM. Results are shown in **FIG. 5D**.

A8

At page 33, the paragraph beginning on line 14:

FIG. 5 illustrates agent concentration dependent cleavage of break light A. To increase the sensitivity for the less efficient reagent Fe^{+2}-EDTA, oligo concentration was increased 10-fold (32 nM), (**FIG 5D**). As a comparison, MPE was also examined at this higher molecular break light concentration, (**FIG 5C**).

A9

At page 33, the paragraph beginning on line 25:

FIG. 6A is a graph of the UV-visible absorption spectra of purified mbp-CalC. The purified mpb-CalC was analyzed in the following solution: 52 μM mpb-CalC; 10 mM Tris-HCl, pH 7.5). The inset shows the results of low temperature (4.3 K) the X-band EPR analysis of CalC. 250 μM mpb-CalC containing 0.5 mol Fe per mol CalC was analyzed in 10 mM Tris-HCl, pH 7.5. The spectrometer settings were as follows: field set = 2050 G; scan range = 4,000G; time constant = 82 s; modulation amplitude =16 G; microwave power = 31 μW; frequency = 9.71 Ghz; gain = 1000; determined spin quantitation = 90 ±10 μM Fe.

A10

At page 34, the paragraph beginning on line 18:

A11

As indicated in **FIG. 6B**, no DNA cleavage was observed in the absence of DTT or calicheamicin (lanes a and b), while efficient cleavage was demonstrated in the presence of DTT and calicheamicin (lane c). As expected, the addition of mbp-CalC completely inhibited calicheamicin-induced DNA cleavage (lane f) while the addition of mbp alone (lane d) as a control, failed to inhibit calicheamicin-induced DNA cleavage. Furthermore, preincubation of mbp-CalC with DTT (not shown), or *apo*-mbp-CalC (lacking the Fe cofactor)(lane e), also failed to inhibit calicheamicin-induced DNA cleavage. However, the addition of Fe^{+2} or Fe^{+3} to the *apo*-mbp-CalC assay could reconstitute CalC activity (lane g). Reconstitution of *apo*-mbp-CalC was accomplished by preincubation with 1 mM $FeSO_4$ (Fe^{+2}) or $FeCl_3$ (Fe^{+3}) prior to the activity assay as previously described.

At page 34, the paragraph beginning on line 31:

A12

Molecular break light probe A was used to assay CalC inhibition of nucleotide cleavage by calicheamicin. As illustrated in **FIG. 8**, CalC directly inhibits calicheamicin-mediated DNA cleavage in the break light assay.

At page 35, the paragraph beginning on line 5:

A13

As is shown in **FIG. 8**, Titration of increasing amounts of CalC into the molecular break light assay in the presence of calicheamicin completely abolishes the cleavage and, thus, the fluorescent signal. Complete inhibition of calicheamicin was achieved with roughly 2-fold excess of CalC. CalC has no effect on esperamicin-induced cleavage of DNA (data not shown).

REMARKS

The specification has been amended to delete references to old Figures 5a and 5b, which were inadvertently omitted from the figures submitted with this application as filed. Old Figures 5a and 5b would have been cumulative to the other disclosures in the application and thus are unnecessary to the full understanding of the invention.

A new set of Drawings is enclosed. Figures 6-9 of the old set of Drawings have been re-numbered as Figures 5-8 to reflect the deletion of references to old